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# Immunohistochemical Analysis of Progesterone Receptor and Proliferating Cell Nuclear Antigen in Canine Inflammatory Mammary Carcinoma

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<http://dx.doi.org/10.5772/54472>

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## 1. Introduction

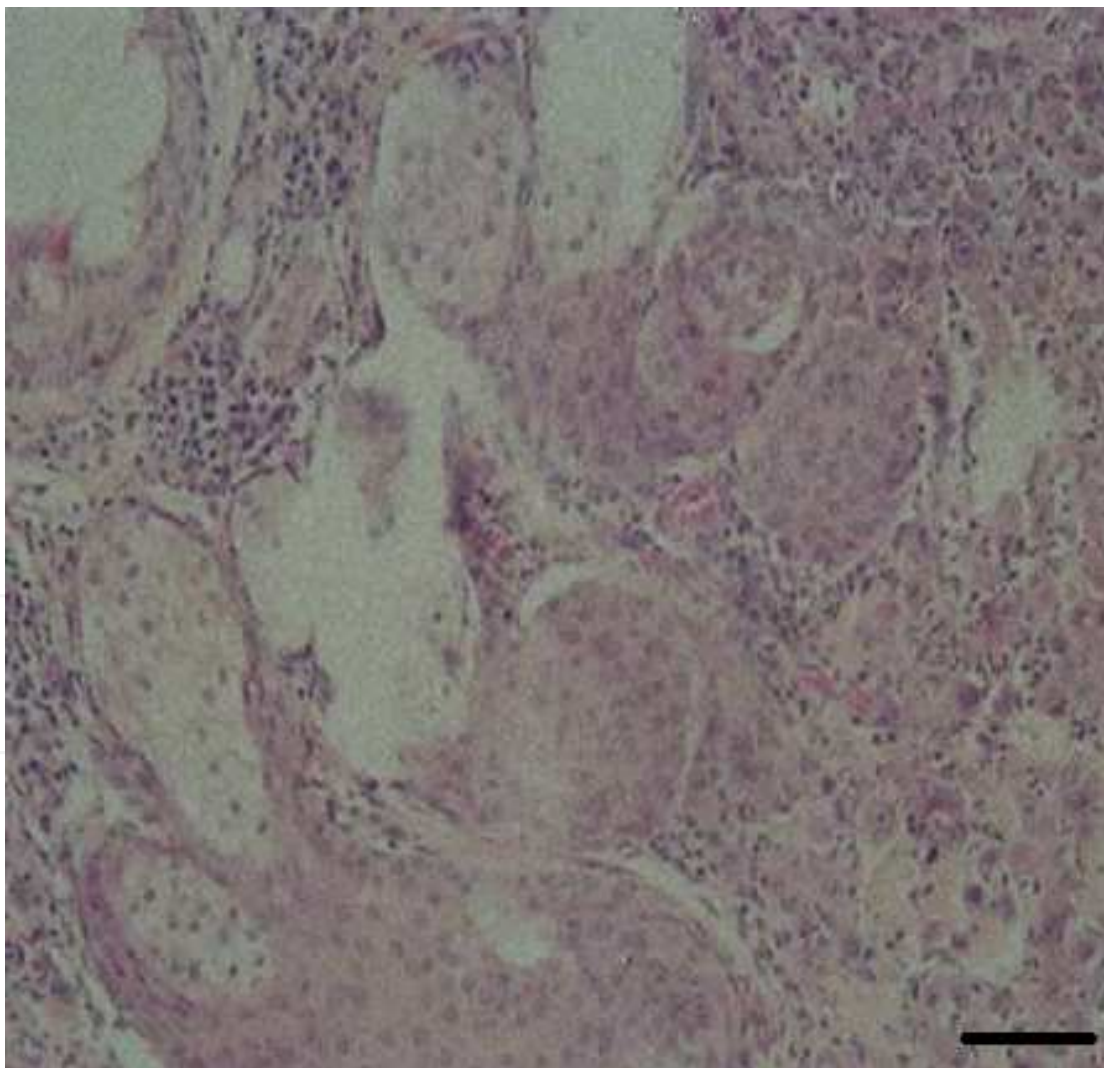
Cancer constitutes a major problem in animal pathology and is a subject of intensive research. Canine mammary tumors are common neoplasms and have been reported to account for up to half of all tumors in female dog [1]. All malignant canine mammary tumors have the potential to metastasize and in general, metastasis tends to occur via the lymphatic system to the inguinal lymph nodes or hematogenously to the lungs or to more distant body sites including the liver, spleen, heart, bone [2]. Canine mammary tumors share many similarities with breast cancer in human beings, including the high prevalence of adenocarcinomas, frequency of metastasis and progressive disease [1].

Inflammatory mammary carcinoma is a special type of locally advanced mammary cancer that is associated with particularly aggressive behaviour and poor prognosis in women - in which case it is termed *inflammatory breast carcinoma* (IBC), and in the dog - in which case it is termed inflammatory mammary carcinoma (IMC) [3-8]. In both species, this uncommon type of tumors corresponds to a locally invasive mammary cancer that can be clinically misdiagnosed as a dermatitis or mastitis owing to its special inflammatory phenotype [9,10]. Although the pathogenesis of the disease remains obscure, some special clinical, genetic, biologic and hormonal characteristics have been found to be specific for inflammatory breast carcinoma and inflammatory mammary carcinoma [9,11-13].

Inflammatory mammary carcinoma is a very specific type of rare, very aggressive and highly metastatic mammary cancer in dogs [7,8]. Clinical features include the presence of pain, erythema, edema and ulceration in the skin of the mammary gland region. These features are similar to symptoms of inflammatory diseases such as mastitis and dermatitis [2,7,8]. Histo-

logically there is evidence of a poorly differentiated carcinoma with extensive evidence of both mononuclear and polymorphonuclear cellular infiltrates and often edema. Dermal lymphatic invasion also can be seen histologically. Clinically, these neoplasms grow and metastasize extremely rapidly and invade lymphatics in the skin, resulting in marked edema and inflammation [14].

Two clinical forms of inflammatory mammary carcinoma have been described in women and dogs [8,9,15-17]: primary and secondary. Primary inflammatory mammary carcinoma occurs suddenly in dogs without previous detection of lesions of mammary tumors while the secondary inflammatory mammary carcinoma accompanies mammary tumors [8]. Secondary inflammatory mammary carcinoma is further classified into two types: postsurgical whenever it develops after surgical excision of a previous mammary tumors, and non-postsurgical when developing from a previous mammary tumor not surgically treated that led to inflammatory mammary carcinoma [9,18,19].



**Figure 1.** *Inflammatory mammary carcinoma (IMC), Hematoxylin-eosin (H&E); Bar = 100µm.*

Inflammatory mammary carcinoma occurs most often in female dogs, but it can strike male dogs too. Most dogs that develop this form of reproductive cancer are females who have never been spayed, or who were spayed after they were two years old. Hormone therapy can also increase your female dog's risk of developing mammary cancer. Spaying your dog before she undergoes her first reproductive cycle at six months of age is the best way to prevent inflammatory carcinoma. Rarely, this type of cancer occurs in male dogs, which also have mammary glands. Dogs who develop a mammary carcinoma will have one or more tumors in their mammary glands. More than half of dogs with this type of cancer develop tumors in more than one mammary gland. Malignant tumors grow quickly, are often irregular in shape, may attach themselves to the surrounding skin or tissue, and may cause painful inflammation and even ulceration of the affected area [8,12,13,20,21].

Inflammatory mammary carcinoma in dogs often causes pain and swelling in the affected area. The tumors may be hard or soft. You will be able to feel them under the skin, and often they may be visible to the naked eye as well. The area will be warm and tender to the touch. It is advised to take tissue biopsies of the tumors to determine if they are cancerous and if they are inflammatory carcinoma. Blood tests and urinalysis can help the practitioner to determine if the cancer has spread, and how it may be affecting other body organs [22].

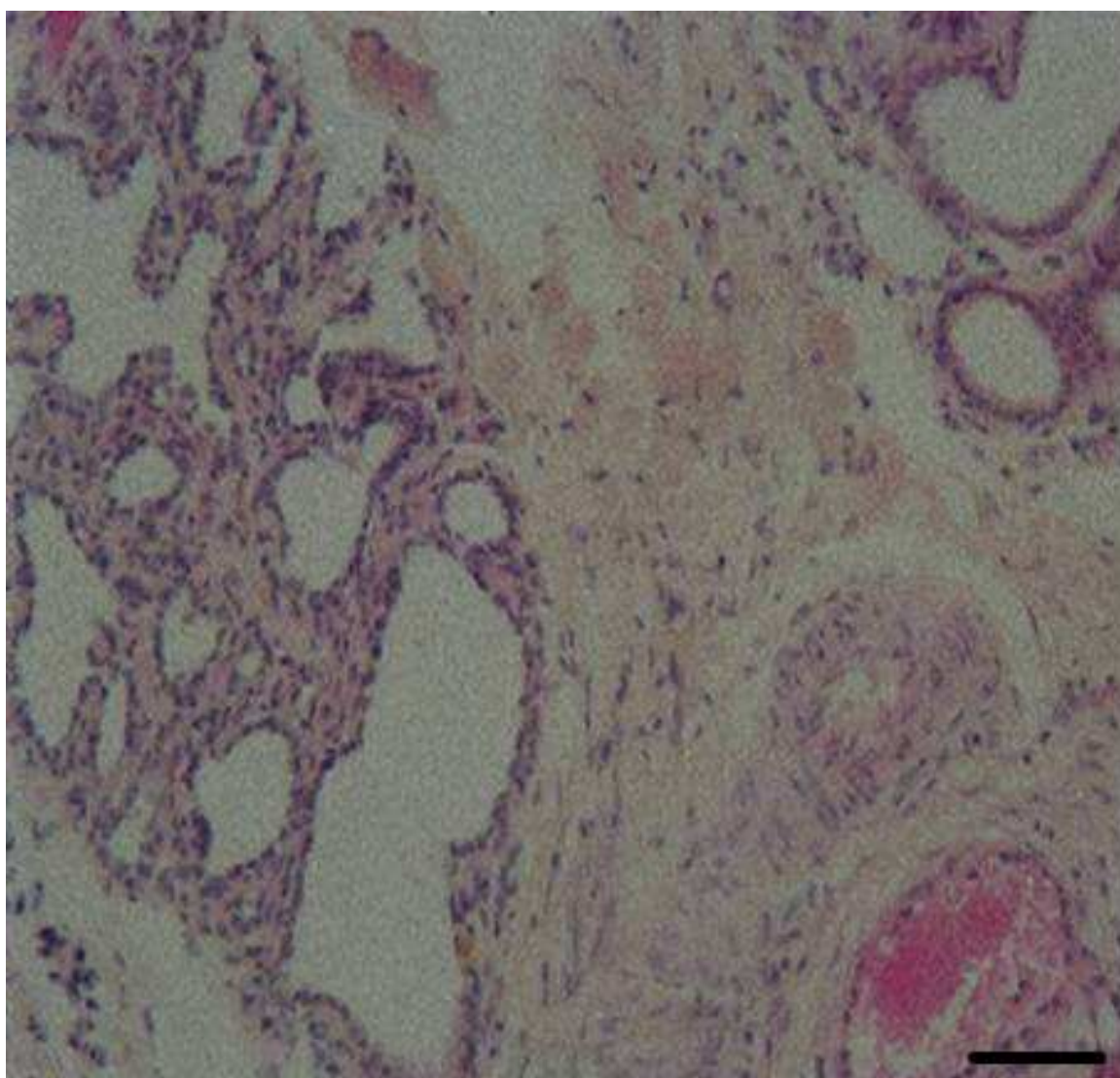
Surgery is sometimes used to remove inflammatory carcinoma tumors, though this is not always advisable in dogs. If the cancer has not yet spread, or if ulceration and infection has occurred, it may be indicated to remove the tumor and affected mammary gland surgically. If the dog has not yet been spayed, your vet may want to perform this procedure as well. Inflammatory mammary cancer in dogs is an aggressive disease that spreads rapidly, and surgery alone often does little to slow or stop its progression [21,22].

The clinician may recommend chemotherapy and radiation therapy to treat your dog's cancer, even if the cancer has not yet spread. The prognosis for inflammatory carcinoma in dogs will depend upon the size of your dog's tumors at the time of diagnosis, and whether or not the cancer has already spread to other parts of the body. Survival time after treatment can range from nine months to two years. Prevent inflammatory carcinoma by having your dog spayed before she is six months old. Dogs who are obese, or who eat diets high in beef and pork, are at an increased risk of developing inflammatory carcinoma [22].

In the clinical evaluation of inflammatory mammary carcinoma observed a strong resemblance to the inflammatory process, so it is often confused with dermatitis [23-25]. Construction of histological inflammatory mammary carcinoma is not uniform - they can be all forms of cancer (Figure 2). Often these are tumors of low maturity. The lymphatic vessels of the skin congestion states of cancer cells. The prognosis is bad.

The purpose of this study was to evaluate of proliferating cell nuclear antigen (PCNA), cytokeratin 19 (CK19), and progesterone receptor (PgR) in two cases of canine inflammatory mammary carcinoma and whether or not these markers might be useful in tumor identification or prognosis.





**Figure 2.** *Inflammatory mammary carcinoma (IMC), Hematoxylin-eosin (H&E); Bar = 100µm.*

## 2. Materials and methods

### 2.1. Materials

A total of 135 samples from canine mammary tumours were used in this study, from which 80 were parafine-embedded archive samples from a period running from 2006 to 2008, and 55 were fresh samples obtained from mastectomy surgery, performed at Warsaw Veterinary Clinics and Small Animal Clinic of the Department of Clinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences – SGGW. Through three years period, only two cases of inflammatory mammary carcinoma (IMC) were diagnosed, which were further confirmed by clinical signs and histopathology were selected for further immunohistochemical studies. Investigated material con-

tained 14 adenomas, 66 complex carcinomas (adenocarcinomas), 47 simple carcinomas (adenocarcinomas), 6 solid carcinomas and 2 inflammatory mammary carcinoma (IMC). The number of cancers with a defined grade amounted to, respectively, 1<sup>st</sup> grade – 48, 2<sup>nd</sup> grade – 39 and 3<sup>rd</sup> grade – 34. Mammary gland neoplasms were excised from female dogs belonging to 9 breeds in the ages between 3 and 16 years. There were 106 mixed-breed dogs, 10 German Shepherd Dogs, 5 Boxers, 3, Rottweilers, 2 Beagles, 6 Yorkshire Terrier and 1 each of the following breed: English Springer Spaniel, Labrador Retriever, Doberman Pinscher.

## 2.2. Methods

Specimens were fixed in 8% buffered formalin, dehydrated and embedded in paraffin. 4µm thick sections were cut from each mammary neoplasm. The sections were mounted onto slides coated with 3-amino-propyltriethoxysilane (Sigma), deparaffinized in xylene and rehydrated in graded ethanol concentrations. Antigen retrieval was performed in 10mM citrate buffer in a microwave oven at 600W for 5 minutes and 300W for 10 minutes. The slides were left to cool at room temperature immersed in the buffer. Thereafter they were washed for 10 minutes in running tap water and rinsing in distilled water. Endogenous peroxidase was quenched by immersion in a solution of 30% hydrogen peroxide and methanol (50 ml of H<sub>2</sub>O<sub>2</sub> and 50 ml of methanol) for 10 minutes. The slides were washed in distilled water then with TRIS (pH 7.4) for 10 minutes, and then incubated with the primary monoclonal antibodies in humid chamber for 1 hour at room temperature. Primary antibody clones and dilutions used for progesterone receptor (PgR), proliferating cell nuclear antigen (PCNA), and for cytokeratin 19 (CK19) presented (table 1).

| Antibody                                  | Type  | Dilution | Antigen Retrieval | Incubation      | Source         |
|-------------------------------------------|-------|----------|-------------------|-----------------|----------------|
| Cytokeratin 19 (CK19)                     | Mab * | 1:50     | HTAR **           | 1 hr, room temp | DakoCytomation |
| Progesterone Receptor (PgR)               | Mab * | 1:50     | HTAR **           | 1 hr, room temp | Novocastra     |
| Proliferating cell nuclear antigen (PCNA) | Mab * | 1:50     | HTAR **           | 1 hr, room temp | DakoCytomation |

(\*) Mab - mouse monoclonal antibody; (\*\*) HTAR – high-temperature antigen retrieval (with 10 mM citrate buffer, pH 6.0).

**Table 1.** The antibodies used for immunohistochemistry

The slides were washed in TRIS for 10 minutes. The EnVision +<sup>TM</sup> System (DakoCytomation) was used for visualization. Visualization was achieved with 3,3'-diaminobenzidine tetrahydrochloride (DAB – DakoCytomation) in Tris-HCl buffer, and after rinsed slides were counterstained with hematoxylin Ehrlich's, dehydrated in graded alcohol concentrations and xylene and closed in DPX mounting medium (Gurr®). For progesterone receptor (PgR), proliferating cell nuclear antigen (PCNA), and cytokeratin 19 (CK19), a canine mammary adenocarcinoma was used as positive control. Computer image analysis and Lucia v. 4.21 software were used for interpretation of the results of PCNA, CK19, PgR, expression; using those facilities we could count the number of neoplastic cells featuring stained cytoplasm and nucleus per 1,000 neoplastic cells. Results were analyzed using the SPSS 12.0 program. To determine whether differences for a few independent traits were significant, Kruskal-Wallis test was used. This test is an equivalent to the test of variance for traits without normal distribution. Two-sided correlations were performed using Spearman correlation test. The differences were deemed statistically significant at  $p \leq 0.05$ .

3. Results and discussion

3.1. Relationship between age of bitches and the grade of malignancy and histological type of tumor of epithelial origin

Dogs were divided into three age groups: <8 years, 8 -12 years and >12 years. In the group of bitches below the age of 8, majority (61.1%) consisted of tumors with the lowest histological grade of malignancy (1<sup>st</sup>). In the oldest group, 1<sup>st</sup> and 2<sup>nd</sup> grade tumors in the 1<sup>st</sup> and 2<sup>nd</sup> accounted for 77.8%. In the entire pool of studied tumors in all age groups, the largest share consisted of tumors with the lowest degree of histological malignancy (40.4%) (Table 2). Assessment of the contribution of individual types of tumors at different ages showed that in bitches younger than 8 years the most common findings were adenomas (21.7%) and complex carcinomas (56.5%) and in those over 12 years simple carcinomas occurred most often (55.0%) (Table 3).

| Year of bitches | Tumor grade |            |            | Total |
|-----------------|-------------|------------|------------|-------|
|                 | I°          | II°        | III°       |       |
| <8 lat (n=18)   | 11 (61.1%)  | 3 (16.7%)  | 4 (22.2%)  | 18    |
| 8-12 lat (n=85) | 30 (36.0%)  | 29 (35.0%) | 26 (30.5%) | 85    |
| >12 (n=18)      | 7 (38.9%)   | 7 (38.9%)  | 4 (22.2%)  | 18    |
| total (n=121)   | 48 (40.4%)  | 39 (32.8%) | 34 (28.1%) | 121   |

Table 2. Incidence of malignancies of various grades in bitches in different age groups

| Year of bitches | Types of tumors |                   |                        |                        | Canine inflammatory mammary carcinoma (IMC) | Total |
|-----------------|-----------------|-------------------|------------------------|------------------------|---------------------------------------------|-------|
|                 | Adenoma         | Carcinoma solidum | Adenocarcinoma simplex | Adenocarcinoma complex |                                             |       |
| <8 lat          | 5 (21,7%)       | 1 (4,3%)          | 4 (17,4%)              | 13 (56,5%)             | 0                                           | 23    |
| 8-12 lat        | 7 (7,8%)        | 4 (4,4%)          | 32 (35,6%)             | 47 (52,2%)             | 2 (2,2%)                                    | 92    |
| ">12 lat        | 2 (10,0%)       | 1 (5,0%)          | 11 (55,0%)             | 6 (30,0%)              | 0                                           | 20    |
| Total           | 14 (10,5%)      | 6 (4,5%)          | 47 (35,3%)             | 66 (49,6%)             | 2 (1,5%)                                    | 135   |

**Table 3.** Occurrence of individual types of epithelial neoplasms in different age groups in bitches

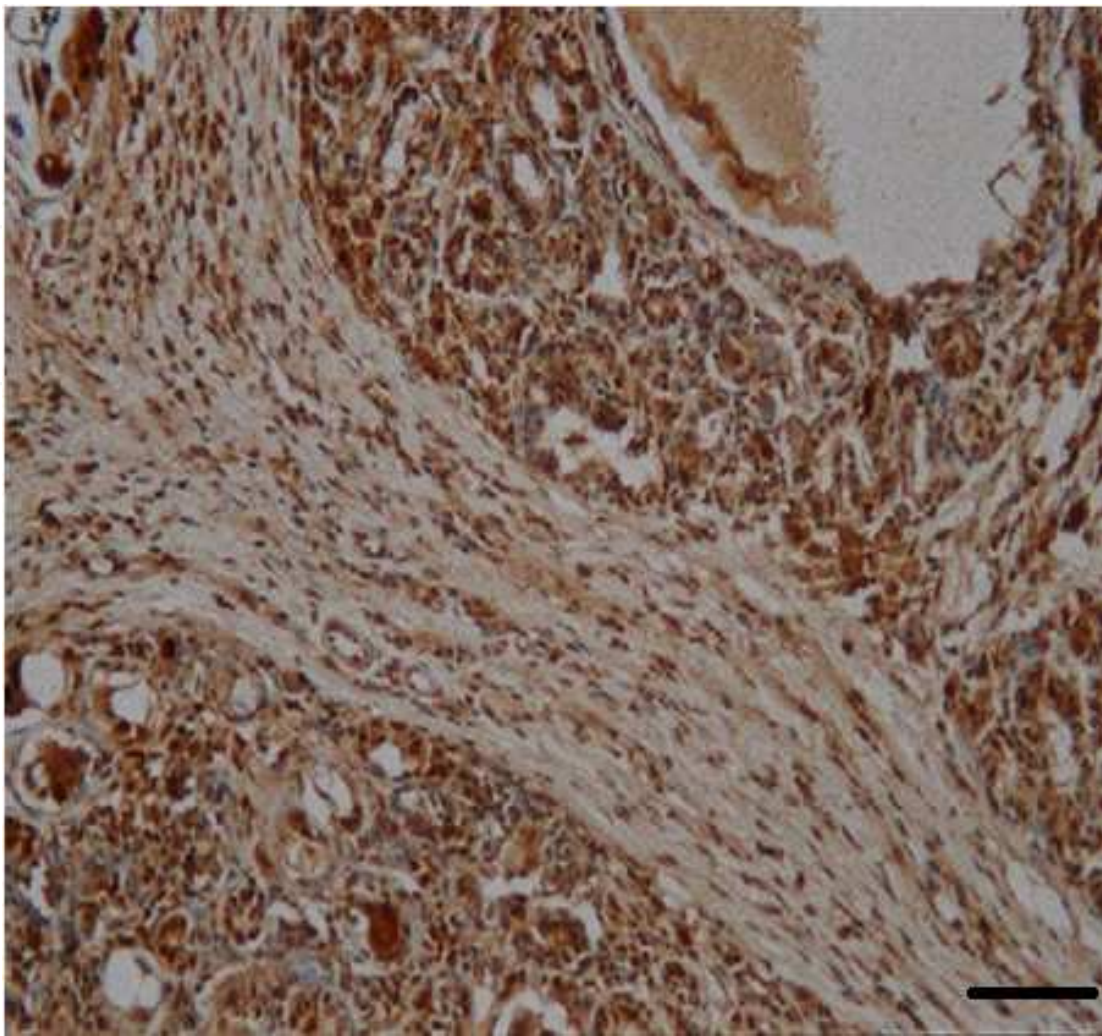
In our study, two inflammatory mammary carcinoma came from the dogs between the ages of 8 and 9 years (case 1 - 8 year, case 2 - 9 year) (Table 2). These dogs had a clinically diagnosis of IMC at the time of initial examination. The most important findings during physical examination included: erythema and warmth, generalized induration of the involved mammary glands, cutaneous nodules affecting the overlying skin, edema of the proximal portion of the hind limbs.

### 3.2. Results of proliferative activity in inflammatory mammary carcinoma

The value of mitotic index differed significantly between types of tumors. The lowest proliferative activity was observed in adenomas, the highest in simple carcinoma, solid carcinoma and inflammatory mammary carcinoma. The highest proliferative activity was found in tumors in the 3<sup>rd</sup> grade of malignancy, the lowest in the tumors in the 1<sup>st</sup> grade. Expression of PCNA was observed in the nuclei of neoplastic cells that have undergone division (Figure 3). Statistical analysis shows significant differences between particular types of tumors. The lowest number of cells exhibiting PCNA expression was observed in adenomas, the highest in the solid carcinoma, simple carcinoma, inflammatory mammary carcinoma and in tumors with the highest histological grade of malignancy (3<sup>rd</sup>).

In the two cases canine inflammatory mammary carcinoma on the basis of H&E histopathology found a high mitotic index (3.4 in the first case in the second case 4.1). PCNA index was similarly and markedly elevated in the two animals: in case 1 inflammatory mammary carcinoma was 82%, in case 2 the inflammatory mammary carcinoma was 85% (Figure 3).





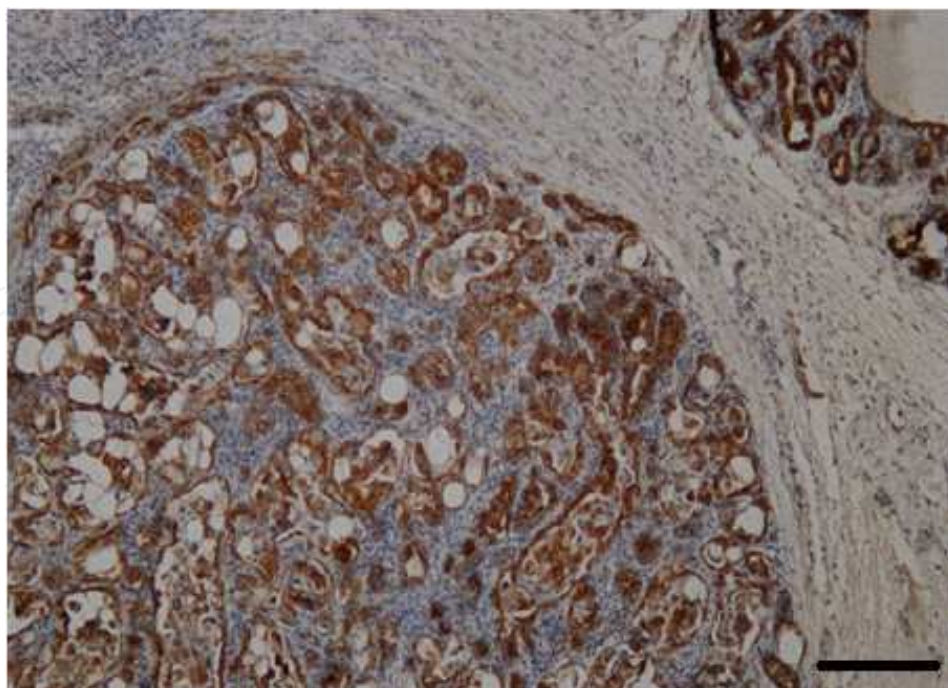
**Figure 3.** *Inflammatory mammary carcinoma – ICM, immunostaining for PCNA in a case of IMC; Bar=100μm*

### 3.3. Results of cytokeratin 19 expression in inflammatory mammary carcinoma

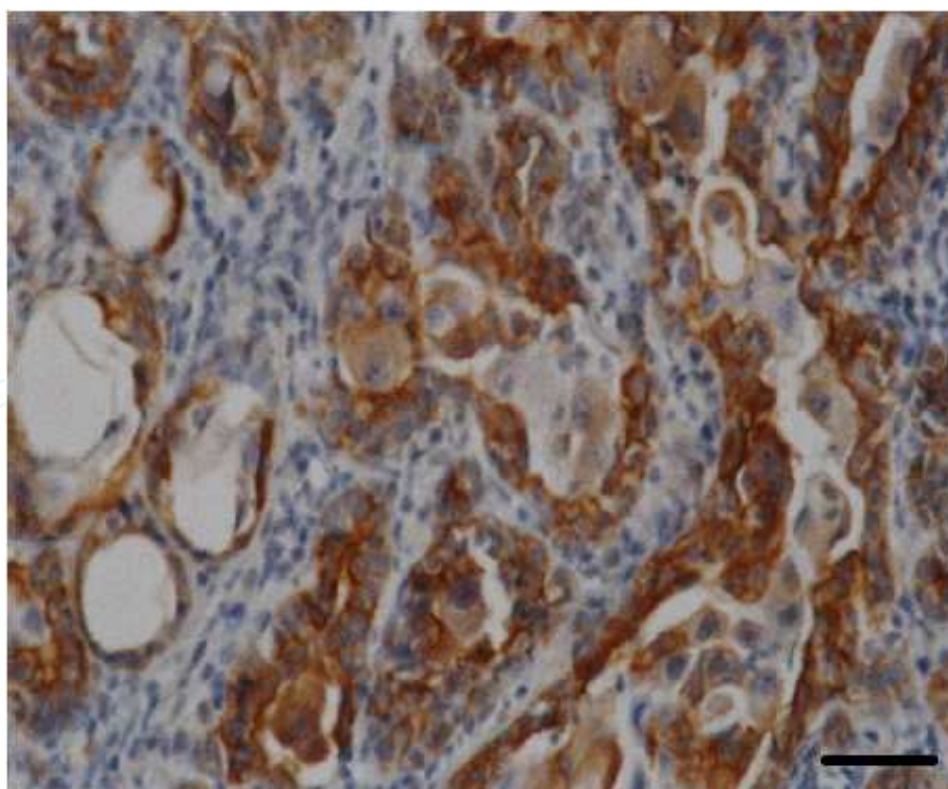
Expression of cytokeratin 19 was observed in the neoplastic cell cytoplasm. Positive CK19 reaction was found in all tumors of epithelial origin. The high expression of CK19 was found also in inflammatory mammary carcinoma (case 1 – intensity level of expression CK19 – 92%; case 2 – intensity level of expression CK19 – 98%) (Figure 4 and 5).

### 3.4. Results of progesterone receptor expression in inflammatory mammary carcinoma

Progesterone receptor expression was detected in the nuclei of tumor cells, but it was also seen in the cytoplasm. Cytoplasmic reaction was considered to be nonspecific. Among all tumors of epithelial origin, expression of progesterone receptors was found in 56 (41.4%), and no reaction was noted in 79 (58.5%). Expression of progesterone receptors was most commonly found in complex cancers (43.9%), simple cancers (42.6%), adenomas (28.6%), while solid cancers rarely expressed them (16.7%). The high expression of progesterone receptors was



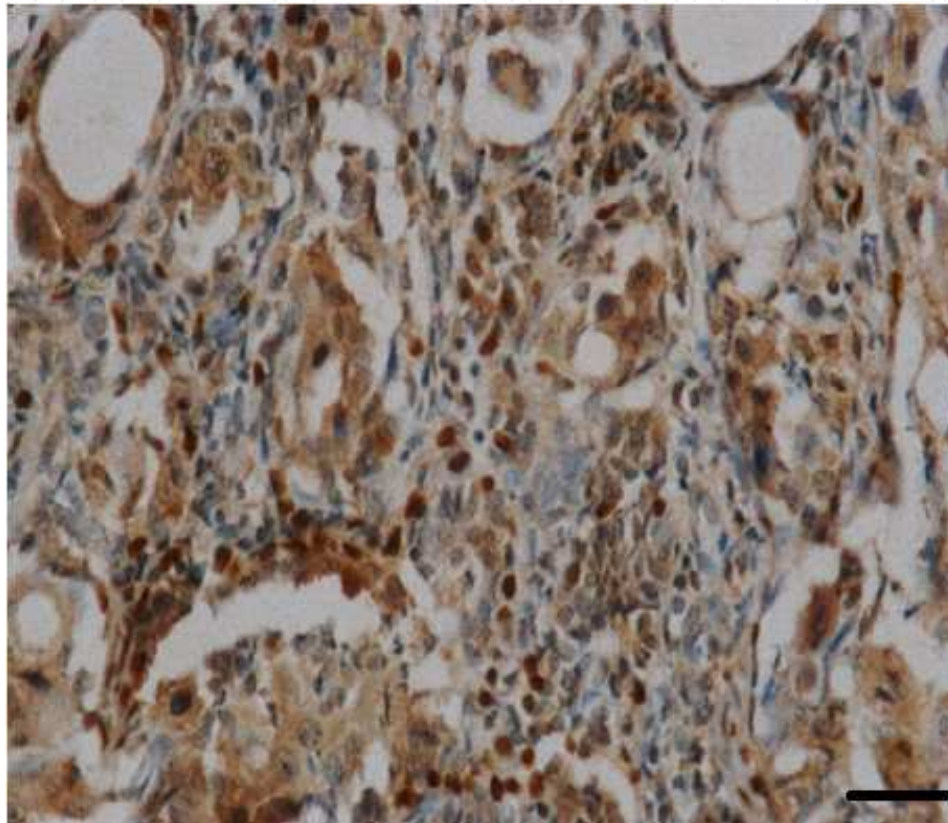
**Figure 4.** *Inflammatory mammary carcinoma – ICM*, immunostaining for cytokeratin 19 (CK19) in a case of ICM; Bar=100  $\mu$ m



**Figure 5.** *Inflammatory mammary carcinoma – ICM*, immunostaining for cytokeratin (CK19) in a case of ICM; Bar=50  $\mu$ m

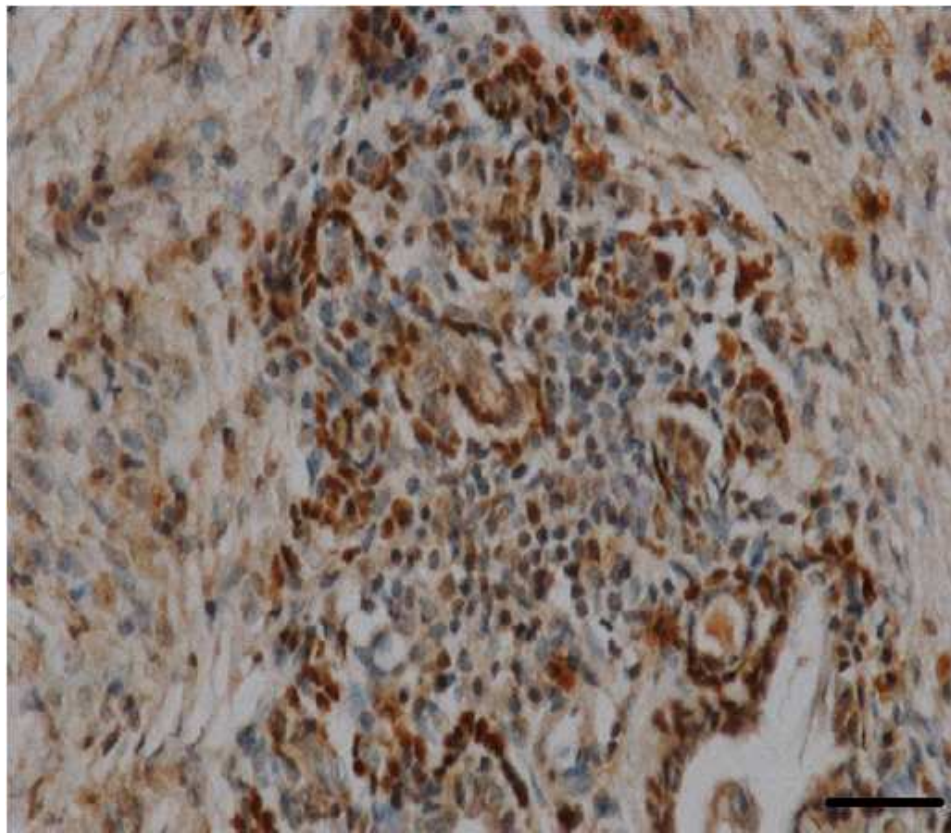


found also in inflammatory mammary carcinoma 2 (100%) (Figure 6 and 7). Analysis of the average number of cells showing positive expression of progesterone receptors reveals that the level of expression increases with the histological grade of malignancy. It was also found that most tumors expressing progesterone receptors came from dogs younger than 8 years. A positive correlation was found between mitotic index and expression of progesterone receptors in specific types of cancer and statistically significant differences between tumor characteristics were demonstrated ( $p=0.042$ ).



**Figure 6.** *Inflammatory mammary carcinoma – ICM, nuclear expression of progesterone receptor (PgR); Bar=50  $\mu$ m*

Clinical signs and histopathological findings are necessary for the accurate diagnosis of inflammatory mammary carcinoma. Our studies have suggested that inflammatory mammary carcinoma is an aggressive malignancy because in our studies, the percentage of PCNA positive cells within the tumor was high in the two cases. An important marker of malignancy is the proliferative activity. Proper evaluation of proliferative activity of tumor cells is crucial for the evaluation of its biological activity and is used in determining the treatment of cancer. In our study, proliferative activity depended on both, the type of tumor and the degree of histological malignancy. The highest values of mitotic index were recorded in simple carcinomas, solid carcinomas and in two cases of inflammatory mammary carcinoma and in tumors with the highest histological grade of malignancy. Similar results were reported for the expression of PCNA. The highest expression of PCNA was seen in solid carcinomas, simple carcinomas and in inflammatory mammary carcinoma and in 3<sup>rd</sup> grade tumors.



**Figure 7.** Inflammatory mammary carcinoma – ICM, nuclear expression of progesterone receptor (PgR); Bar=100  $\mu$ m

In our studies among all tumors of epithelial origin, expression of progesterone receptors was found in 41.4%. Amorim *et al.* (2008) in their study found no expression of PgR in 9 cases inflammatory mammary carcinoma [26]. However, Pen *et al.* (2003) found in their study high positive immune reaction to PgR in inflammatory mammary carcinoma [12]. The high positive immune reaction to PgR in canine inflammatory mammary carcinoma suggests, the possible involvement of special endocrine mechanisms in inflammatory mammary carcinoma development.

During the three years in the research material collected was diagnosed only two cases of canine inflammatory mammary carcinoma, therefore it can be said that canine inflammatory mammary carcinoma is a rare cancer in dogs [8]. Perez *et al.* showed that inflammatory mammary carcinoma is rare in dogs (17,7% of all cases within 4 years) [8]. Yet Susaneck *et al.* [13] claimed that the incidence of canine inflammatory mammary carcinoma has doubled in the past 15 years [7]. Pena *et al.* [12,13] in their research work diagnosed 33 of canine inflammatory mammary carcinoma cases in a 5 years period.

All these authors described this type of cancer as a rare, unusual and different. Authors investigated the relationship between clinical and histopathological characteristics of canine inflammatory mammary carcinoma specifying nuclear antigen expression of PCNA, and expression of progesterone receptor [7,8,12,13].

## 4. Conclusion

Based on the literature and our own experience it can be concluded that canine inflammatory mammary carcinoma is a rare tumors with poor prognosis. Our research suggests that inflammatory mammary carcinoma is an aggressive malignancy, with a tendency to metastasize at an early stage.

## Acknowledgements

Study was carried out at the Institute of Pathology, Department of Pathology and Veterinary Diagnostics, Faculty of Veterinary Medicine, Warsaw School of Life Sciences, 159C Nowoursynowska Street, 02-766 Warsaw. Research was conducted as a part of a doctoral dissertation, partially funded by a grant from the Ministry of Science and Information Technology, No N30800632/0667.

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